Kasetsart J. (Nat. Sci.) 40 : 370 - 381 (2006)

Identification of the Conserved Sequences at the Species Level of Odontoglossum Ringspot Virus and Other Members of Tobamovirus by Its Coat Protein Sequence Analysis

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ABSTRACT

A synthetic 1056 base pair at 3′-terminal region of odontoglossum ringspot virus (ORSV) genome was determined by its amino acid sequence compositions. The deduced coat protein (CP) sequence of ORSV was compared with 32 available published data of tobamovirus CPs. The relationship of amino acid composition of tobamovirus CPs fell into three major clusters similar to the tobamovirus subgroups previously classified. The amino acid sequence within ORSV group showed highly conserved, almost 96-100% identical homology, but was distinct from the other tobamovirus subgroups. However, the topography of the phylogenetic tree showed that the orchid-infecting, crucifer-infecting (85-100% identity), some tomato-infecting (81-94% identity) and tobacco-infecting virus strains (95-98% identity) formed tight clusters which were mostly correlated with the host range preferences of the viruses. The application of the program clustalW to amino acid data alignment classified and identified the conserved sequence and genetic variability at the species level of each member of tobamovirus subgroups by its coat protein sequence analysis. According to the high degree of conservation in ORSV CP composition, it was successful in detecting virus infecting broad length of orchid host plants showing severe symptoms or appearing symptomless by RT-PCR with ORSV CP specific primers.

Key words: tobamovirus, odontoglossum ringspot virus, coat protein

INTRODUCTION

Odontoglossum ringspot virus (ORSV) or tobacco mosaic virus-orchid strain (TMV-0) was first isolated from *Odontoglossum grande* orchid (Jensen and Gold, 1951). More than 20 genera of orchids have been reported to be infected by ORSV showing such symptoms as color-breaking in flower and a variety of symptoms in leaves ranging from latent through chlorotic streaks or mosaic to necrosis (Buchen-Osmond *et al.*, 1988). ORSV has been isolated from *Dendrobium* orchids in Thailand since 1971 (Samutsin and Sutabutara, 1971). ORSV and cymbidium mosaic virus (CymMV) are known as co-infection viruses, which are widely distributed and affect the economic value of orchids, especially oncidium. ORSV belongs to *Tobamovirus* genus with 300×18 nm stiff rod particle. The virion contains a single molecule of

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Received date : 13/03/04 Accepted date : 30/06/06

positive sense linear ssRNA, 6.6 kb in size (Paul, 1975). The complete nucleotide sequence has been previously reported by Ryu and Park (1995) and Chng *et al*. (1996). The 3′-terminal region encoded for coat protein (CP) was found highly conserved among various kinds of orchids cultivated in Asia (Ajjikuttira *et al*., 2002). The *Tobamovirus* genus has 13 species. ORSV was classified into tobamovirus subgroup1 together with solanaceae-infecting tobamoviruses by its related serology (Park *et al*., 1990; Paul, 1975). Cucurbit-infecting and legume-infecting viruses were classified into subgroup 2, while tobamoviruses infecting crucifer and a few others were placed into subgroup 3 (Wetter, 1986; Lartey *et al*., 1994). Although the genetic data of those tobamovirus species are available, the ORSV genome sequence of ORSV Thai strain and phylogenetic relationships within the genus have not been analyzed. Therefore, the 3′–erminal genomic sequence of ORSV was identified and compared its CP sequence with those of other tobamoviruses previously reported. The determined CP amino acid composition could be used for tobamovirus classification. Additionally, the specific primers designed from ORSV CP were comparatively evaluated for ORSV and CymMV detection in orchids using RT-PCR.

MATERIALS AND METHODS

Virus purification and RNA extraction

Virus infecting oncidium orchid was isolated from *Chenopodium quinoa*. The particles of virus causing disease were detected under electron microscope. Then, the virus was mainly propagated in *Chenopodium amaraticolors.* Virus particles were extracted from infected leaves of *C. amaraticolors* by chloroform-carbon tetrachloride and purified by sucrose density gradient according to the method of Samutsin and Sutabutara (1971). The genomic RNA was extracted by sodium dodecyl sulfate-proteinase K treatment as described by Both and Air (1979).

Complementary DNA synthesis and cloning

Approximately 0.5 microgram of viral genomic RNA was synthesized to first stranded of cDNA and then amplified using the one-step polymerase chain reaction as described by Globet *et al*. (1989). Both ORSV-3′ primer: 5′CGGTA CCTGGGCCTCTACCC 3′ corresponding to nt 6597-6609 with *Kpn*I site and ORSV-5′ primer: 5′CCTATGGCTAGGGCTCTC3′ corresponding to nt 5482-4599 with *Xba*I were added into PCR reaction mixture together with *Taq* DNA polymerase (Gene Amp Kit, Perkin-Elmer Cetus) and AMV reverse transcriptase (Promega Corporation). The mixture was subject to DNA Thermal Cycler Model 480 with the 30 cycles of PCR. The viral RNA was first synthesized to first strand of cDNA by reverse transcriptase at 42°C for 15 min, then denatured at 99°C for 30 s and cooled down at 5°C for 5 min. Then, the cDNAs were amplified with the 30 cycles of denatured at 94°C for 30 s, annealed at 55°C for 1 min, extended at 72°C for 2 min and then completed the amplified fragments at 72°C for 2 min. The PCR products were analyzed by 1% agarose gel electrophoresis.

The DNA fragments from PCR products were ethanol precipitated and digested with *Kpn* I and *Xba* I (Biolab). PCR products were excised from agarose gel and extracted using Gene Clean Kit (Bio101). The extracted DNA was ligated into the pBluescript KS(-) and finally transformed to *Escherichai coli* strain XL1-blue. The colony selection, plasmid preparations and restriction analysis were performed as described by Sambrook *et al*. (1989). DNA sequencing reactions were performed using ABI PRISM™ Dye Terminator Cycle Sequencing Ready Kit with AmpliTaq® DNA Polymerase, FS (Perkin-Elmer). To complete the sequence, the selected clone of pOONC21 was subject to nested deletion by using exonuclease III of Erase-a-Base^{R} (Promega Corporation) as described by the manufacturer.

Computer analysis

The total DNA sequences were compiled by the DNA connecting program HIBIO DNASIS™ (Hitachi). The primary structure analysis of nucleic acid and deduced amino acid sequences were performed with sequence utilities described in the website http://searchlauncher.bcm.tmc.edu. Blastn and blatsp programs in the website http://www.ncbi.nlm.nih.gov/ were used for a database searching. A program, ClustalW at the website http://iubio.bio.indiana.edu/treeapp/treeprint-form.html, was used to assess the correlation between amino acid sequences by alignment and to represent the similarities as dendrograms. Sequences for multiple alignment were retrieved from EMBL and GenBank databases.

ORSV detection by RT-PCR and Southern analysis

Reverse transcription PCR (RT-PCR) and Southern blot hybridization were performed to simultaneously detect ORSV and CymMV infecting orchids. Total RNA were extracted from random selected 8 orchid cultivars, including *Cattleya, Dendrobium, Mokara, Oncidium, Phaelaenopsis, Rhynchostylis, Spathoglottis, and Vanda*, by the hot phenol/LiCl method as described by Verwoerd *et al*. (1989). The RNAs were reversely transcribed into cDNA using Ready-To-Go™ Your-Prime First-Strand Beads (Pharmacia Biotec). The cDNA were amplified by RT-PCR method using two pairs of primer that included specific primer for ORSV CP as described above and the specific primer for CymMV CP gene, i.e. CymMV-3′ primer: 5′TTGGATCC$(T)_{12}$ at the 3′-end of genomic RNA and CymMV-5′ primer 5′ CCTATGGCTAGGGCTCTC3′ at nt 5441-5456 (Srifah *et al.*, 1996). The cDNAs were analyzed on a 1.0% agarose gel and subsequently transferred to nylon membrane. Hybridization was performed at 65°C with ORSV and CymMV probe which amplified from positive clone pOONC21 and pCONC9 (personal communication), respectively, and labeled as suggested by the manufacturer's protocol (Gene Image, Random Primer Labelling, Amersham Biosciences).

RESULTS AND DISCUSSION

Virus purification and RNA extraction

High concentration of virus particles were purified from infected *Chenopodium amaranticolor* showing chlorotic spots after 7-10 days inoculation. The particles of viruses causing disease were 300 nm stiff rod particles under electronmicroscope (data not shown). The viral genomic RNA was successfully extracted from the purified virus and about 1.08 mg/ml was obtained.

Viral gene cloning and sequencing

The single-stranded RNA genome of ORSV at 3′ terminal was reverse transcribed and amplified to a single double stranded DNA. One microgram of about 1,056 nt PCR product was digested with *Kpn*I and *Xba*I and then cloned in pBluescript KS (-) plasmid. More than twenty clones were positives. One selected clone, pOONC21 was fully sequenced from both ends and subcloning into pOONC15 and pOONC16 by *Pst*I-*Hinc*II digestion and pOONC 21.1 and pOONC 21.2 by nested deletion method. The total data of 5,702 nt was obtained from those clones and used to compile the genomic sequence of the 3′-terminal of ORSV using connecting program of HIBIO DNASIS™ (Hitachi). Most of the 1,056 nt sequence was obtained as an overlapping set in both orientations from independent clones. Computer analysis of its sequence revealed one major open reading frame (ORF) coding for a protein with predicted M_r of 17,725.89 (18K). The ORF started at nucleotide 159 and terminated with UAA at nucleotide 633, encoding a protein of 158 amino acid residues (18K). The composition of the nucleotide sequence and deduced amino acid sequence are shown in Figure 1. However, the ORF closest to the 3′-terminal of the genome encodes the coat protein (CP). The amino acid

composition of the encoded protein, coat protein, predicted from the gene sequence is similar to that determined by Matthews (1992). A search using blastp program showed that this protein was similar to the ORSV CP Singapore isolate (Accession Q84136).

Comparisons of the coat protein and other tobamoviruses

The sequence of deduced ORSV CP Thai isolate (ORSVT) was compared and aligned with the related sequences from 32 other tobamoviruses retrieved from the GenBank and EMBL sequence database. The variation at each position of the aligned protein was evaluated with ORSV CPs. The highest identity was found among ORSV CPs. The deduced ORSVT CP consisted of a sequence that was identical to that of ORSV CP (Q84136) Singapore isolate and 1-2 amino acid residues differed from four other reported ORSV isolates. The percentage homology of ORSV CPs, namely ORSVS, ORSVJ (P03678) Japanese isolate, ORSVKE3 United States isolate, ORSVF (A60023) French isolate and ORSVC (Q84122) Korean isolate were 100%, 99%, 99%, 98% and 96%, respectively, compared with ORSVT Thai isolate of oncidium orchid. Interestingly, the amino acid sequence comparison revealed that ORSVC Korean isolate of cymbidium orchid contained five amino acid changes at the positions 69, 76, 80, 100 and 139 in tyrosine (Y), histidine (H), asparagine (D) glutamic acid (E) and threonine (T), respectively.

The CPs of tobamovirus in the same host species were almost the same in length of about 155-163 amino acid residues, except Frangipani

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tctagacttaatagccgtaataaattaaattatgaaaatggtgatagtgatgtt
ggattagtgttgtagtagatgatgttatggttggtaatggtgttagtgatatt
cgtattgatgatgattgtgagtcatttgacgcacaatctgattcgtattgaat
    159 atgtcttacactattacagacccgtctaagctggcttatttaagc
        M  S  Y  T  I  T  D  P  S  K  L  A  Y  L  S
    204 tcggcttgggctgaccccaattcactaatcaacctttgtaccaat
        S  A  W  A  D  P  N  S  L  I  N  L  C  T  N
    249 tctctgggtaatcagttccaaacacaacaagctcgaacaactgtt
        S  L  G  N  Q  F  Q  T  Q  Q  A  R  T  T  V
    294 caacagcagtttgctgatgtttggcagccggttcctactttgacc
        Q  Q  Q  F  A  D  V  W  Q  P  V  P  T  L  T
    339 agtaggttccctgcaggcgctggttacttcagagtttatcgctat
        S  R  F  P  A  G  A  G  Y  F  R  V  Y  R  Y
    384 gatcctatattagatcctttaataactttcttaatgggtacttttt
        D  P  I  L  D  P  L  I  T  F  L  M  G  T  F
    429 gatactcgtaatagaataatcgaggtagaaaatccgcagaatccg
        D  T  R  N  R  I  I  E  V  E  N  P  Q  N  P
    474 acaactacggaaacattagacgcaactcgtagagttgatgatgca
        T  T  T  E  T  L  D  A  T  R  R  V  D  D  A
    519 actgtagcaataagatctgcaataaataatctattaaacgagtta
        T  V  A  I  R  S  A  I  N  N  L  L  N  E  L
    564 gttaggggaactggcatgtacaatcaagtctcatttgagacgatg
        V  R  G  T  G  M  Y  N  Q  V  S  F  E  T  M
    609 tctggacttacttggacctcttcctaatcatatttaggaaaataa
        S  G  L  T  W  T  S  S  *
cgttgatagtgttgaactatccgtggtgcatacgataatgcatagtggttatc
cctccacttaaatcgaagggtttttcactgcggatatgtaggtttcctacggt
gaatataaaacttatatcccgttgtgtacacgatagtacatagtgtttatccc
tccacttaaatcgaagggtttttgtgtcagacgcgtgtaaggagtggtcaacct
tacgacacatttaaaataatgcgtccgtggtgcatacgataagagtggtcaac
cttacgacacatttaaaataatgcgtccgtggtgcatacgataatgcatagtg
tttgtccctccacttaaatcgaagggttgtgtatatggaccatgcggataagt
tatactggtgcagtataacccgttatacacataaaattatgagggattcgaat
tccccttacctcgggtagaggcccaggtaccg
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Figure 1 The nucleotide and deduced aa sequences of the 3′-terminal region of the ORSV cDNA isolated from oncidium orchid.

mosaic virus (FrMV) posted eight extra amino acids at the C-terminus (Table 1 and Figure 2). The pairwise comparisons of the aligned sequence showed that the deduced CP had a sequence that was almost identical to ORSV CPs and some similar to those thirty-two other tobamoviruses, so the ORSV CP cluster was distantly related to those of other tobamoviruses. The similar amino acid sequences were seen throughout their length, and only two particular variable regions were found in the stretch of 9-16 amino acid residues near the N-terminus and a few amino acids at position 66. The SHMV CP and ORSV CP contained an extra proline (P) and two glycine and tyrosine (GY) or glycine and histidine GH) amino acid residues (Figure 2). Two motifs of QTQ and DDA blocks were found at C-terminus and N-terminus of all tobamovirus CPs.

The clustalW of ORSV CP alignment classified tobamoviruses into three groups based on their amino acid compositions. The conserved sequence regions could identify the genetic variability at the species level of each member of tobamovirus subgroups; solanaceae-infecting and orchid-infecting virus in subgroup 1; crucifer and some other-infecting virus in subgroup 2; and cucurbit and legume-infecting virus in subgroup 3. The most conserved sequence was found in subgroup 1, even though some sequence regions of ORSV CPs differed from the others sequences in the same subgroup (Figure 2). The percent homology levels of amino acid sequences of tobamovirus CPs of the virus infecting cucurbit and other hosts compared with TMV CP tobamovirus type strain were 31-35 % of CGMMV, KGMMV, and ZGMMV; 40-49% of ChRMV, CrMV, FrMV, RMV, SHMV, TMVHR, TVCV, YoMV; and ranging from 71-74% of ORSV CPs, whereas those solanaceae strains of ObPV, PaMMV, TMGMV and TMVU2 shared 59-69%; TMVER, TMVKO, TMVOM, TMVRA, and TMVV-NC shared 80-85%; and PMMV, ToMV, ToMVK2, TMVDA, TMVL, shared 91%-98% (Table 1). Two motifs of QTQ and DDA blocks were found at C-terminus and N-terminus of all tobamovirus CPs.

Comparisons of the amino acid sequences of the 33 tobamovirus CPs including ORSVT have provided a phylogenetic framework to examine and compare some of the other viruses. In the dendrogram, the relationship of tobamovirus CPs fell into three major clusters (Figure 3). The orchid-infecting (96-100% aa identity), crucifer-infecting (85-100% aa identity) and some tomato (81-94% aa identity) and tobacco-infecting strains (95-98% aa identity) form tight clusters, which were mostly correlated with the host range preferences of the viruses, whereas cucurbit-infecting (43-77% aa identity) pepper-infecting (61-87% aa identity) and the others of tobacco-infecting strains were distantly related. The most notable feature of the phylogenetic tree of the coat protein composition revealed that almost all of the genus *Tobamovirus* members, except TMVHR, were classified in the same subgroup. According to serologically cross-reacting by ELISA and immunoelectron microscopic assay, ORSV was classified into subgroup 1 viruses together with solanaceous-infecting TMV, ToMV, PMMV and TMGMV (Dubs and Regenmortel, 1990; Park *et al.*, 1990; Paul, 1975). When RT-PCR was used for detecting five tobamoviruses group 1 which were serologically related, the ORSV was different from the other tobamoviruses **(**Letschert *et al.*, 2002). The tobamoviruses subgroup 2 contained cucurbit-infecting CGMMV and legume-infecting SHMV and subgroup 3 comprised of crucifer-infecting viruses and RMV **(**Wetter 1986; Lartey *et al.*, 1990). These studies showed that the phylogenetic topology of tobamovirus CP amino acid composition was similar to the topology of MP sequences, a relationship previously reported by Chng *et al.* (1996) and Ryu and Park (1994). It was clear that the assembly of tobamovirus subgroup using amino acid composition of CPs could classify the species in genus tobamovirus.

Table 1 List of tobamoviruses used and their coat protein amino acid sequence homology between TMV tobamovirus and other thirty-two reported tobamoviruses.

Virus code	Origin/virus name	Subgroup	Accession #	aa size	%aa sequence identity
Odontoglossum ringspot virus (TMV-infecting orchid)					
ORSVC	Korean isolate	1	Q84122	158	72
ORSVKE3	US isolate	1	unknown	158	73
ORSVF	French isolate	1	A60023	158	72
ORSVG	German isolate	1	CAD22088.1	158	72
ORSVJ	Japanese isolate	1	P03678	158	73
ORSVS	Singapore isolate	1	Q84136	158	74
ORSVT	Thai isolate	1	-	158	74
Tobacco mosaic virus strains (TMV-infecting tobacco)					
TMV	Tobacco mosaic virus	1	AAD20290.1	159	100
TMVDA	TMV-Dahelmense	1	6505767C	156	98
TMVER	TMV-ER strain	1	P03573	159	81
TMVKO	TMV-Kokubu&O strain	1	VCTMKO	155	85
TMVRA	TMV-Rakkyo strain	1	Q98747	159	80
TMVV	TMV-vulgare strain	1	AF273221	155	84
TMGMV	Tobacco mild green mosaic /TMV-U2 strain	1	VCTMU2	156	69
TMVHR	TMV-HR strain	3	VCTMHR	156	45
Tobacco mosaic virus strains (TMV-infecting tomato)					
TMVL	TMV-tomato strain	1	VCTMDB	158	98
TMVOM	TMV-tomato strain	1	P03571	155	84
ToMV	Tomato mosaic virus/tomato	1	AF411922-1	159	91
ToMVK2	Tomato mosaic virus K2 strain	1	Z92909	156	96
Tobacco mosaic virus strains (TMV-infecting pepper)					
ObPV	Obuda pepper virus /pepper	3	D13438	160	59
PaMMV	Paprika mild mottle virus /paprika	3	X72586	161	61
PMMV	Pepper mild mottle virus /pepper	1	AF103777	156	97
Tobamovirus-infecting crucifer					
ChRMV	Chinese rape mosaic virus	3	AAB60601.1	157	48
CrMV	Crucifer mosaic virus	3	BAA28951.1	157	49
TuVCV	Turnip vein-clearing virus	3	AAC02785.1	157	49
YoMV	Youcai mosaic virus/crucifer	3	NC-004422	157	48
Tobamovirus-infecting cucurbit					
CGMMV	Cucumber green mottle mosaic	2	BAA87622.1	161	35
KGMMV	Kyuri green mottle mosaic virus	2	CAB65681.2	161	31
ZGMMV	Zucchini green mottle mosaic	2	CAB64668.1	161	32
Tobamovirus-infecting other hosts					
FrMV	Fragipany mosaic virus/*Plumeria* sp.	2	AF165884	174	40
RMV	Ribgrass mosaic virus/*Plantago* sp.	3	AAB08579.1	157	47
SHMV	Sunn-hemp mosaic virus/legume	2	P03581	163	40

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ORSVKE3         MSYTITDPSKLAYLSSAWADPNSLINLCTNSLGNQFQTQQARTTVQQQFADVWQPVPTLT 60
ORSVJ           MSYTITDPSKLAYLSSAWADPNSLINLCTNSLGNQFQTQQARTTVQQQFADVWQPVPTLT 60
ORSVT           MSYTITDPSKLAYLSSAWADPNSLINLCTNSLGNQFQTQQARTTVQQQFADVWQPVPTLT 60
ORSVS           MSYTITDPSKLAYLSSAWADPNSLINLCTNSLGNQFQTQQARTTVQQQFADVWQPVPTLT 60
ORSVF           MSYTITDPSKLAYLSSAWADPNSLINLCTNSLGNQFQTQQARTTVQQQFADVWQPVPTLA 60
ORSVC           MSYTITDPSKLAYLSSAWADPNSLINLCTNSLGNQFQTQQARTTVQQQFADVWQPVPTLT 60
ORSVG           MSYTITDPSKLAYLSSAWADPNSLINLCTNSLGNQFQTQQARTTVQQQFADVWQPVPTLT 60
TMVDA           MSYSITSPSQFVFLSSVWADPIELLNVCTSSLGNQFQTQQARTTVQQQFSEVWKPFPQST 60
TMVL            -SYSITSPSQFVFLSSVWADPIELLNVCTSNLGNQFQTQQARTTVQQQFSEVWKPFPQST 59
TMV             MSYSITSPSQFVFLSSVWADPIELLNVCTNSLGNQFQTQQARTTVQQQFSEVWKPFPQST 60
TOMK2           MSYPITSPSQFVFLSSVWADPIELLNVCTNSLGNQFQTQQARTTVQKQFSEVWKPFPQST 60
PMMV            MSYSITSPSQFVFLSSVWADPIELLNFGTNSLGNQFQTQQARTTVQQQFSEVWKPFPQST 60
ToMV            MSYAITSPSQFVFLSSAWADPVELINICTNSLGNQFQTQQARTTVQQQFSEVWKPFPQST 60
TMVER           MSYNITTPSQFVFLSSAWADPLELINLCTNALGNQFQTQQARTVVQRQFSEVWKPSPQVT 60
TMVRA           MSYNINTPSQFVFLSSAWADPIELINLCTNALGNQFQTQQARTVVQRQFSEVWKPSPQVT 60
TMVV-NC         MSYSITTPSQFVFLSSAWADPIELINLCTNALGNQFQTQQARTVVQRQFSEVWKPSPQVT 60
TMVOM           MSYSITTPSQFVFLSSAWADPIELINLCTNALGNQFQTQQARTVVQRQFSEVWKPSPQVT 60
TMVKO           MSYSITTPSQFVFLSSAWADPIELINLCTNALGNQFQTQQARTVVQRQFSEVWKPSPQVT 60
TMGMV           MPYTINSPSKFVYLSSAYADPVQLINLCTNALGNQFQTQQARTTVQQQFADAWKPVPSIT 60
TMVU2           MSYTINSPSQFVYLSSAYADPVELINLCTNALGNQFQTQQARTTVQQQFADAWKPSPVMT 60
KGMMV           MSYSTSGIRSLPAFAKSFYPFYDVYNLLVSAQGGALQTQNGKDILRESLTGLLTSVASLN 60
ZGMMV           MPYSTSGIRSLPAFSKSFFPYLELYNLLITNQGAALQTQNGKDILRESLVGLLSSVASPT 60
CGMMV           MAYNPITPSKLIAFSASYVPVRTLLNFLVASQGTAFQTQAGRDSFRESLSALPSSVVDIN 60
SHMV            MAYSIPTPSQLVYFTENYADYIPFVNRLINARSNSFQTQSGRDELREILIKSQVSVVSPI 60
ObPV            MPYTVTSPSQLVYFGSVWADPITFIDLCTVALGNQFQTQNARTTVQQQFSDLFKTVPTRT 60
PaMMV           MPYTVSSPNQLVYFGSVWADPIALIDLCTVSLGNQFQTQNARTTVQQQFSDLFKTVPTRT 60
YoMV            MVYNITSSNQYQYFAAMWAEPTAMLNQCVSALSQSYQTQAARDTVRQQFSNLLSAIVTPN 60
ChRMV           MVYNITSSNQYQYFAAMWAEPTAMLNQCVSALSQSYQTQAARDTVRQQFSNLLSAIVTPN 60
CrMV            MSYNITNSNQYQFFAAVWAEPIAMLNQCVSALSQSYQTQAARDTVRQQFSNLLSAIVTPN 60
TMVHR           -SYNITNSNQYQYFAAVWAEPTPMLNQCVSALSQSYQTQAGRDTVRQQFANLLSTIVAPN 59
RMV             MSYNITNSNQYQYFAAVWAEPTPMLNQCVSALSQSYQTQAGRDTVRQQFANLLSTIVAPN 60
TuVCV           MSYNITNPNQYQYFAAVWAEPIPMLNQCMSALSQSYQTQAARDTVRQQFSNLLSAVVTPS 60
FMV             MSYTNISTTNIIYFARSYIPGVDFIDLIISGLGQSFQTQSARDEFRSQLIGSYKVVVSKT 60
                  *      .   :   :     . :      .   *** .:  .:  :

ORSVKE3         SRFPAGAGYFRVYRYDPILDPLITFLMGTFDTRNRIIEVE--NPQNPTTTETLDATRRVD 118
ORSVJ           SRFPAGAGYFRVYRYDPILDPLITFLMGTFDTRNRIIEVE--NPQNPTTTETLDATRRVD 118
ORSVT           SRFPAGAGYFRVYRYDPILDPLITFLMGTFDTRNRIIEVE--NPQNPTTTETLDATRRVD 118
ORSVS           SRFPAGAGYFRVYRYDPILDPLITFLMGTFDTRNRIIEVE--NPQNPTTTETLDATRRVD 118
ORSVF           SRFPAGAGYFRDYRYDPILDPLITFLMGTFDTRNRIIEVE--NPQNPTTTETLDATRRVD 118
ORSVC           SRFPAGAGHFRVYRYEPILEPLITFLMGTFDTRNRIIEVR--NPQNPTTTETLDATRRVD 118
ORSVG           STLPAGAGYFRVYRYDPILDPFLTFLLGTCDTRNRIIEVE--NPQNPTTTETLDATRRVD 118
TMVDA           VRFPGDV--YKVYRYNAVLDPLITALLGTFDTRNRIIEVE--NQQSPTTAETLDATRRVD 116
TMVL            VRFPGDV--YKVYRYNAVLDPLITALLGAFDTRNRIIEVE--NQQSPTTAETLDATRRVD 115
TMV             VRFPGDV--YKVYRYNAVLDPLITALLGAFDTRNRIIEVE--NQQSPTTAETLDATRRVD 116
TOMK2           VRFPGDV--YKVYRYNAVLDPLITALLGTFDTRNSIIEVE--NRQSPTTAETLDATRRVD 116
PMMV            VRFPGDV--YKVYRYNAVLDPLITALLGSFDTRNRIIEVE--NQQNPTTAETLDATRRVD 116
ToMV            VRFPDIV--FKVYRYNAVIDPLITALLGTFDTRNRIIEVE--NQQSPTTAETLDATRRVD 116
TMVER           VRFPDRD--FKVYRYNAVLDPLVTALLGAFDTRNRIIEVE--NQANPTTAETLDATRRVD 116
TMVRA           VRFPDSD--FKVYRFNAVLDPLVTALLGAFDTRNRIIEVE--NQANPSTAETLDATRRVD 116
TMVV-NC         VRFPDSD--FKVYRYNAVLDPLVTALLGAFDTRNRIIEVE--NQANPTTAETLDATRRVD 116
TMVOM           VRFPDSD--FKVYRYNAVLDPLVTALLGAFDTRNRIIEVE--NQANPTTAETLDATRRVD 116
TMVKO           VRFPDSD--FKVYRYNAVLDPLVTALLGAFDTRNRIIEVE--NQANPTTAETLDATRRVD 116
TMGMV           VRFPASD--FYVYRYNSTLDPLITALLNSFDTRNRIIEVD--NQPAPNTTEIVNATQRVD 116
TMVU2           VRFPASD--FYVYRYNSTLDPLITALLNSFDTRNRIIZVB--BZABPTTAVPIBTZZRVD 116
KGMMV           SRFPAN--EFFVWSRESRIAAIIDSLLSALDSRNRAIEVE--NPSNPSTGEALNATKRND 116
ZGMMV           SQFPSG--VFYVWSRESRIAALIDSLFGALDSRNRAIEVE--NPSNPSTGEALNAVKRND 116
CGMMV           SRFPDA--GFYAFLNGPVLRPIFVSLLSSTDTRNRVIEVV--DPSNPTTAESLNAVKRTD 116
SHMV            SRFPAEP-AYYIYLRDPSISTVYTALLQSTDTRNRVIEVE--NSTNVTTAEQLNAVRRTD 117
ObPV            NRFNDGENGFRVFRYNSTLDPLISALMNSFDTRNRIIEVD--NPANPNTSEVASATQRVD 118
PaMMV           IRFSDGENGFRVFRYNSTLDPLITALLNSFDTRNRIIETE--NPANPNTAEIASATQRVD 118
YoMV            QRFPEAG--YRVYINSAVLKPLYESLMKSFDTRNRIIETE--EESRPSASEVANATQRVD 116
ChRMV           QRFPEAG--YRVYINSAVLKPLYESLMKSFDTRNRIIETE--EESRPSASEVANATQRVD 116
CrMV            QRFPETG--YRVYVNSAVLKPLYEALMKSFDTRNRIIETE--EESRPSASEVANATQRVD 116
TMVHR           QRFPDTG--FRVYVNSAVIKPLYEALMKSFDTRNRIIQTE--EQSRPSASQVANATQRVD 115
RMV             QRFPDTG--FRVYVNSAVIKPLYEALMKSFDTRNRIIETE--EESRPSASEVANATQRVD 116
TuVCV           QRFPDTG--SRVYVNSAVIKPLYEALMKSFDTRNRIIETE--EESRPSASEVANATQRVD 116
FMV             VRFPENT--IYLWANNPTIRPLLHAVFQALDTRNRIIEVESTNVVNPTSSETREATRRVD 118
                  :         :   . : ..   :: : *:**  * .        .:     :  * *
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ORSVKE3         DATVAIRSAINNLLNELVRGTGMYNQVSFETISGLTWTSS---------------- 158
ORSVJ           DATVAIRSAINNLLNELVRGTGMYNQVSFETISGLTWTSS---------------- 158
ORSVT           DATVAIRSAINNLLNELVRGTGMYNQVSFETMSGLTWTSS---------------- 158
ORSVS           DATVAIRSAINNLLNELVRGTGMYNQVSFETMSGLTWTSS---------------- 158
ORSVF           DATVAIRSAINNLLNELVRGTGMYNQVSFETMSGLTWTSS---------------- 158
ORSVC           DATVAIRSAINNLLNELVRGNGMYNQVSFETMSGLTWTSS---------------- 158
ORSVG           DATVAIRSAINNLLNELVRGTGMYNQVSFETMSGLTWTSS---------------- 158
TMVDA           DATVAIRSAINNLVNELVRGTGLYNQNTFESMSGLVWTSA---------------- 156
TMVL            DATVAIRSAINNLVNELVRGTGLYNQNTFESMSGLVWTSAPAS------------ 158
TMV             DATVAIRSAINNLVNELVRGTGLYNQNTFESMSGLVWTSAPVS------------ 159
TOMK2           DATVAIRSAINNLVNELVRGTGLYNQNTFESMSGLVWTSA---------------- 156
PMMV            DATVAIRSAINNLVNELVRGTGLYNQNTFESMSGLVWTSA---------------- 156
ToMV            DATVAIRSAINNLVNELVRGTGFYNQSTFESMSGLAWTSAPAS------------ 159
TMVER           DATVAIRSAINNLIVELIRGTGSYNRSSFESSSGLVWTSGPAT------------ 159
TMVRA           DATVAIRSAINNLIVELTRGTGSYNRSSFESSSGLVWTSSPAT------------ 159
TMVV-NC         DATVAIRSAINNLIVELIRGTGSYNRSSFESSSGLVWTS---------------- 155
TMVOM           DATVAIRSAINNLVVELIRGTGSYNRSSFESSSGLVWNS---------------- 155
TMVKO           DATVAIRSAINNLVVELIRGTGSYNRSSFESSSGLVWTS---------------- 155
TMGMV           DATVAIRASINNLANELVRGTGMFNQAGFETASGLVWTTSPAT------------ 159
TMVU2           DATVAIRASINNLANELVRGTGMFNQAGFETASGLVWTTT---------------- 156
FMV             DATVAVRSQLQLLFDALSGGSGLYDRKAFEDASGLVWEEAAAVGTAGTSGTGTTTA 174
KGMMV           DASTAAHNDIPLLLAALNDGVGVFDSASFESAFGLTWTASATSSK---------- 161
ZGMMV           DASTAAHNDIPQILSALNEGAGVFDRASFESAFGLVWTAGSSTSS---------- 161
CGMMV           DASTAARAEIDNLIESISKGFDVYDRASFEAAFSVVWSEATTSKA---------- 161
SHMV            DASTAIHNNLEQLLSLLTNGTGVFNRTSFESASGLTWLVTTTPRTA---------- 163
ObPV            DATVNIRACINNLMNELVRGTGMMNTASFETVSNLTWTTTTT------------- 160
PaMMV           DATVSIRACINNLMNELARGTGMLNTVSFETISNLTWTTAATT------------ 161
YoMV            DATVAIRSQIQLLLNELSNGHGLMNRAEFEVL--LPWATAPAT------------ 157
ChRMV           DATVAIRSQIQLLLNELSNGHGLMNRAEFEVL--LPWATAPAT------------ 157
CrMV            DATVAIRSQIQLLLSELSSGHGLMNRAEFEVL--IPWATAPAK------------ 157
TMVHR           DATVAIRSQIQLLLNELSNHGGYMNRAEFEAI--LPWTTAPAT------------ 156
RMV             DATVAIRSQIQLLLNELSNGHGYMNRAEFEAI--LPWTTAPAT------------ 157
TuVCV           DATVAIRSQIQLLLSELSNGHGYMNRAEFEAL--LPWTTAPAT------------ 157
                **:.  :  :  :   :    .  :   **    : *
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Figure 2 Multiple alignment of amino acid sequences of twenty-three CP sequences of tobamoviruses. Bold and underline indicated conserved regions in tobamoviruses subgroup 1 and subgroup 3. Asterisks indicated identity in all the sequences. Colons indicated amino acid differences in two or more isolates. Dashes indicated absence of amino acids.

The shared amino acid sequences in the coat proteins of those ORSV isolates from each part of the world are highly conserved. This may imply that there is a slow evolution in the CP gene of ORSV. Although the ORSV isolates of Korea, Singapore and Taiwan infected different species of orchids such as *Aranda, Epidendrum, Cattleya, Cymbidium, Oncidium, Phaelaenopsis, Ryncovanda* and *Spathoglottis*, this revealed that the isolates were not clustered according to geographic origin and those ORSV CPs were genetically stable (Ajjikuttira *et al*., 2002). However, the tobamoviruses which infect solanaceous and cucurbits plants are different, especially those pepper, tomato and tobacco-infecting strains with symptom variation. This might indicate that the only viral CPs of tobacco-infecting strains of tobamoviruses underwent increasing evolutionary change. The new tobacco virus strains adapted to plant species of other families.

RT-PCR and Southern blot hybridization

Both ORSV and CymMV were successfully co-detected in 3 out of 5 orchid varieties by RT-PCR. The expected length of the amplified fragments corresponding to 3′-terminus of ORSV and CymMV region would be 1130 and 788 bp, respectively. Three samples, one from *Mokara* gave faint bands while those from *Oncidium* and *Catleya* yielded clear DNA bands of approximately 1,130 bp which was the expected

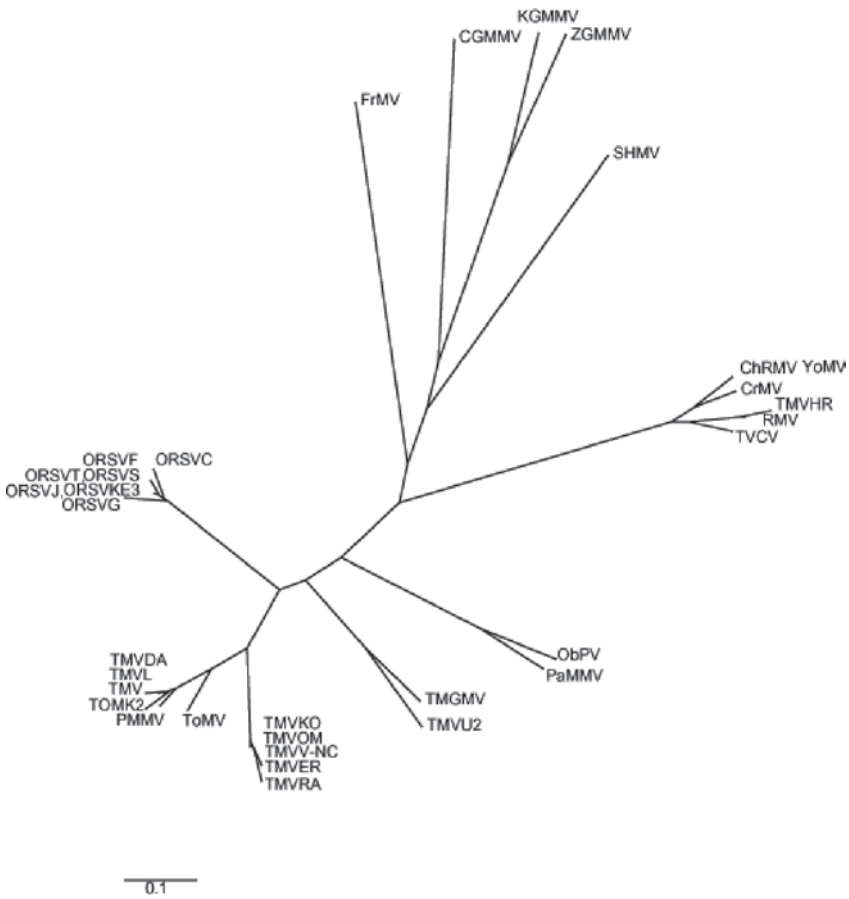

Figure 3 Phylogenetic tree of the amino acid sequences of the coat protein of thirty- three tobamoviruses. The tree was constructed by the Neighbor-Joining method using CLUSTAL W. The scale bar indicated 0.1 substitutions/site.

size for an ORSV specific band. A fragment of 788 bp was observed in 4 individual reactions using CymMV primers and the total RNA extracted from infected *Mokara, Oncidium, Rhynchostylis* and *Catleya* (Figure 4A). Some of the CymMV amplified bands were faint. One distinguished band of a 550 bp in size was amplified from *Rhynchostylis.* To confirm the RT-PCR detection, Southern blot hybridization with ORSV and CymMV probe was used to evaluate the amplified fragements. The hybridization analysis showed that all of 1,130 bp bands and 788 bp bands corresponding to the PCR products hybridized by ORSV (Figure 4B) and CymMV probe (Figure 4C), respectively. The specific primers complementary to the 5′ region of the targeted ORSV CP gene and the 3′ end of the viral genome in this method was proved to be an efficient tool for rapid screening and subsequent cloning of the viral genome using RT-PCR techniques. Main advantages of this method were high accuracy of virus detection, allowing to easily adapted the use of multiplex PCR with those two sets of specific primers to detect CymMV and ORSV in orchid plants.

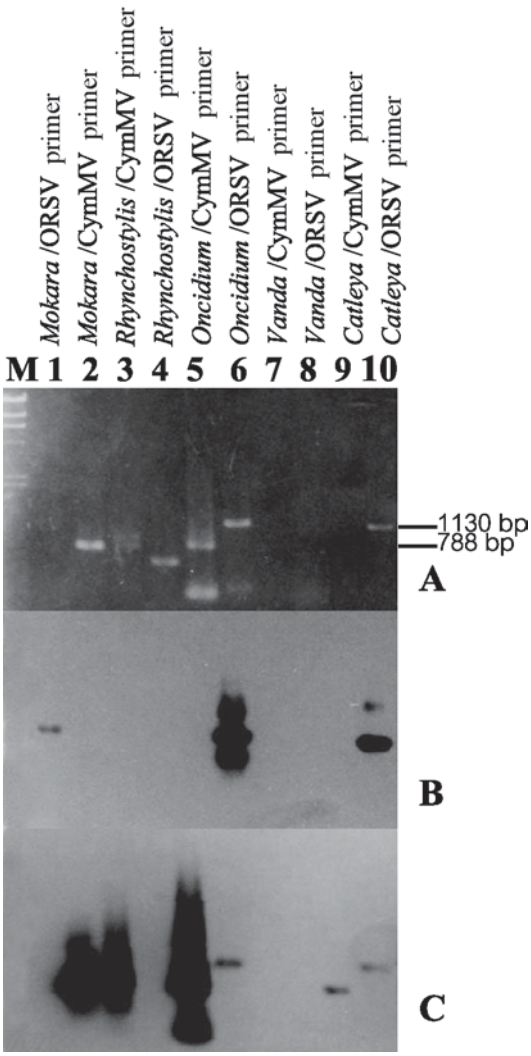

Figure 4 (A) DNA fragments amplified by RT-PCR from total RNA using specific primers to detect CymMV and ORSV in *Mokara* (1, 2), *Rhynchostylis* (3, 4), *Oncidium* (5, 6), *Vanda* (7, 8), *Catleya* (9,10) orchids. (B) The 1130 bp fragments amplified with ORSV primers hybridized with ORSV CP gene probe. (C) The amplified 788 bp fragments obtained by CymMV specific primers were hybridized with CymMV CP gene probe. M is a 1 kb DNA marker.

Further tests were done, using ELISA and IEM assays, to detect ORSV and CymMV in those PCR tested orchid samples and additional other 3 orchid species (Table 2). ORSV positive IEM and ELISA results were obtained from infected *Oncidium* and *Catleya* orchids with severe symptoms but not from symptomless *Mokara, Rhynchostylis, Vanda, Dendrobium, Phalaenopsis,* and *Spathoglottis* orchids, except that RT-PCR was able to detect ORSV from *Mokara.* Furthermore, comparisons showed that RT-PCR with specific primers was more sensitive than ELISA tests as RT-PCR was able to detect CymMV from 7 out of 8 orchids, *Mokara, Rhynchostylis, Oncidium, Catleya, Dendrobium, Phalaenopsis* and *Spathoglottis* while ELISA gave negative results in *Phalaenopsis* and *Spathoglottis.* Both RT-PCR and ELISA tests gave negative results with the *Vanda* samples. This result indicated that ELISA failed to detect ORSV and CymMV in infected orchids that were symptomless. The minimum amount of template ORSV RNA required for detection was reported to be 10 fg, and RT-PCR was a 10^3 times more sensitive than ELISA (Ryu and Park, 1995). The RT-PCR technique used in these experiments was more sensitive than ELISA technique. Therefore RT-PCR technique was introduced as plant quarantine protocal for routine plant virus diagnosis of CymMV and ORSV infecting orchid in Thailand.

ACKNOWLEDGEMENTS

This work was financially supported by Kasetsart University Research and Development Institute (KURDI), Bangkok, Thailand. We would like to thank Dr. Sylvia German for providing ORSV CP clone (pKE3). The authors wish to thank Dr. Martin Huehne and Dr. Paiboon Vattanaviboon for critical reading of our manuscript and helpful suggestions.

Table 2 Detection of ORSV and CymMV in eight orchid species by immunoelectron microscopy (IEM), ELISA, RT-PCR with specific primer and Southern blot (SB).

Host name	CymMV				ORSV			
	RT-PCR 788 bp	SB	ELISA	IEM	RT-PCR 1130bp	SB	ELISA	IEM
Mokara	+	+	+	+	+/-	+	-	-
Rhynchostylis	+	+	+	+	-	-	-	-
Oncidium	+	+	+	+	+	+	+	+
Vanda -	-	-	-	-	-	-	-	
Catleya	+	+	+/-	+	+	+	+/-	+
Dendrobium	+	+	+	+	-	-	-	-
Phalaenopsis	+	+	-	-	-	-	-	-
Spathoglottis	+	+	-	-	-	-	-	-

Note + = positive result; - = negative result

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